EVIDENCE FROM SEA URCHIN–SAND DOLLAR HYBRID EMBRYOS FOR A NUCLEAR CONTROL OF ALKALINE PHOSPHATASE ACTIVITY

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Several investigators (Hultin, 1948a, 1948b; Bohus Jensen, 1953; Tyler and Metz, 1955) have shown that hybridization in echinoids can be facilitated by treatment of the eggs with trypsin. A recent review by Moore (1949) has covered the problem of inheritance in hybrid plutei of echinoids. It seemed of interest to determine the activity of two enzymes (acid and alkaline phosphatase) in the developing embryos of two separate species and of the hybrids. In the hybrids it was hoped that it would be possible to assess the relative role of the nuclei and the cytoplasm in directing the synthesis and activity of these two enzymes.

Alkaline phosphatase activity rises rapidly from gastrulation on to the pluteus stage (Mazia *et al.*, 1948; Gustafson and Hasselberg, 1950), while acid phosphatase maintains a constant level of activity during sea urchin development according to Gustafson and Hasselberg (1951). However, the latter authors have noted a slight but definite rise in acid phosphatase activity in the sea urchin *Paracentrotus lividus*.

Paternal antigens arising by the late blastula stage in sea urchin hybrids have been demonstrated serologically by Harding, Harding and Perlmann (1954), but little information exists dealing with quantitative chemical differences in hybrids. Brachet (1954) has expressed the belief that the nucleus exerts a very important control over enzymes of the microsomes, as evidenced from work upon nucleated and enucleated halves of amoebae.

Utilizing the Gomori–Takamatsu cytochemical technique Krugelis (1947b) has noted that nuclear alkaline phosphatase activity increases from gastrulation to the pluteus stage while that of the cytoplasm declines. In the unfertilized egg the weak staining reaction for the enzyme is primarily located in the cytoplasm (Krugelis, 1947a) and this is borne out by Mazia *et al.* (1948) who utilized biochemical methods and found equal activity in nucleated and enucleated halves of unfertilized eggs separated by the Harvey method. The Gomori-Takamatsu technique has been criticized by Novikoff (1951) and Johansen and Linderstrom-Lang (1952); these authors believe that diffusion obscures the accurate localization of the calcium phosphate precipitate and that nuclei may adsorb the precipitate. Novikoff *et al.* (1950) isolated rat liver nuclei and found less alkaline phosphatase activity in that fraction as compared to the cytoplasm, and Stern *et al.* (1952), utilizing a non-aqueous medium for isolating nuclei (Behren's technique), obtained similar results for nuclei isolated from the thymus gland. However, Dounce (1943) found a higher con-

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centration of alkaline phosphatase in the isolated nuclei from rat liver as compared to the whole tissue. Danielli (1953) expresses the belief that the Gomori-Takamatsu cytochemical technique can successfully localize alkaline phosphatase in the cell and this would mean that a number of workers who cite a nuclear localization of alkaline phosphatase (Danielli, 1946; Krugelis, 1947b; Brachet and Jeener, 1948; and Bradfield, 1950) are correct. The question of localization of this enzyme is an open one and it was hoped the method of hybridization might help to clarify it.

It was originally planned to hybridize reciprocally *Strongylocentrotus purpuratus* and *Strongylocentrotus franciscanus* (see Moore, 1943, for a discussion of maternal and paternal inheritance in this cross), but it was found that there was essentially no difference in acid and alkaline phosphatase activity between the two species. Also it is known that when *S. franciscanus* eggs are fertilized by *S. purpuratus* sperm, development is blocked at the late blastula stage (Moore, 1943). Hybridization of *S. purpuratus* and *Dendraster excentricus* is advantageous in that these genera are more distantly related; they differ markedly in their speed of development, and the cross can be made reciprocally.

Materials and Methods

Eggs and sperm of *Dendraster* and *S. purpuratus* were obtained by injection of 0.5 *M* KCl (Tyler, 1949) into the body cavity. The eggs of *Dendraster* could be fertilized by the usual dilute suspension of *S. purpuratus* sperm used in the homologous crosses. In order successfully to fertilize *S. purpuratus* eggs with *Dendraster* sperm (Tyler and Metz, 1955), the eggs were placed in a 0.05% trypsin (crystalline-lyophilized preparation from Worthington Biochemical Sales Co.) solution for ten minutes and the eggs were then washed several times with sea water to remove the trypsin. They were then fertilized with an amount of 1% sperm (1 drop of dry sperm/5 cc. sea water) which was in forty-fold excess of the volume of sea water containing the eggs. After the eggs were left in this concentrated sperm solution for 30–40 minutes (with frequent agitation of the solution), the eggs were then washed four or five times to remove the excess sperm and cultured in a slowly rotating four-liter flask which floated in a tank of running sea water. The temperature of this running sea water was usually about 10° C. With this procedure about 40% of the eggs were successfully fertilized and hatched swimming blastulae could be separated from the unfertilized eggs.

On the first, second, third, and fourth days after fertilization embryos were collected by centrifugation and were washed several times with the appropriate buffer. These washes were carried out rapidly so as to prevent any loss of the enzyme. For the acid phosphatase assays this was a 0.1 *M* sodium acetate-acetic acid buffer of pH 5.32; for the alkaline phosphatase assays a 0.1 *M* sodium veronal-HCl buffer (0.0015 *M* $MgCl_2$) of pH 9.0 was utilized. For a given assay the embryos were suspended in 4 cc. of the acid or alkaline buffer and disintegrated in an all-glass homogenizer which was kept immersed in an ice bath during homogenization. Then a two-cc. aliquot of the brei was added to two cc. of the substrate (0.1 *M* sodium β glycerophosphate); for the acid phosphatase assays the pH of the substrate solution was adjusted to 6.0. One of the mixtures of brei plus substrate was immediately inactivated by the addition of 0.8 cc. of 60% trichloracetic acid while the other was allowed to incubate at 25° C. for a two-hour period at which time a simi-

lar amount of trichloracetic acid was added. The control and experimental samples were then centrifuged at approximately 10,000 G and the trichloracetic acid supernates were collected. Phosphorous assays were made upon these samples utilizing the Fiske-Subbarow technique (1925) and the control values subtracted from the experimental ones. The trichloracetic acid precipitates were each suspended in one cc. of water and total nitrogen determinations (Umbreit *et al.,* 1948) were made upon aliquots from these samples. Acid or alkaline phosphatase activity is stated as the ratio of total micrograms of phosphate released in a two-hour period divided by the total micrograms of trichloracetic acid-insoluble nitrogen. Acid-insoluble nitrogen is a good standard for these measurements since it remains fairly constant during early development.

Results

Preliminary assays of acid phosphatase in developing embryos of *Strongylocentrotus purpuratus* and *S. franciscanus* showed the activity of this enzyme to be essentially similar in the two species, and preliminary determinations of acid phosphatase in *Dendraster* gave values within this same range. Since acid phosphatase could not be used quantitatively to distinguish *S. purpuratus* from *Dendraster,* this enzyme was not assayed in the hybrids nor were further determinations made in the homologous species. The series of determinations for any given batch of eggs seemed to indicate a slight increase in activity of acid phosphatase during development, but the average values only substantiated an increased activity from the blastula to the gastrula stage.

The activity of alkaline phosphatase was quite different in *Dendraster* and *S. purpuratus,* rising quite sharply for the sand dollar embryos and much more slowly for the sea urchin embryos (Fig. 1). The points in Figure 1 represent two series of experiments, all of which were conducted under similar conditions and gave excellent reproducibility. It was of interest to see if the activity of this enzyme in the hybrids was characteristic of the maternal or the paternal species, or if it might be intermediate between the two. If the latter alternative held true, this would be a quantative indication of a nuclear control of alkaline phosphatase activity, whereas a maternal rate of activity would indicate a cytoplasmic independence of this enzyme. It was not expected that a paternal activity rate would be found.

Hybridization of *Dendraster* eggs by *S. purpuratus* sperm results in normal development up through gastrulation but in the experiments reported here the embryos then became abnormal and gave rise to so-called spherical plutei. These "plutei" did not swim actively and died within a few days. It is believed that the low alkaline phosphatase activities which were found here are a reflection of the poor condition of the embryos. Even if these embryos had developed normally, this would not have been the best cross to ascertain nuclear or cytoplasmic dependence of the enzyme since the maternal species (*Dendraster*) normally has a high alkaline phosphatase activity by the pluteus stage. An intermediate value in this case would be a decrease from the normal activity of the maternal species and might be ascribed to lowered vitality of the hybrids. However, in the *S. purpuratus* ♀ × *Dendraster* ♂ hybrids an intermediate value would be an increase from the normal activity of the maternal species and could clearly be ascribed to the effect of the paternal nuclear material during development. It was fortunate that these hybrids

developed perfectly normally. Some were kept as long as 12 days after fertilization and they were still perfectly healthy plutei at that time.

The *S. purpuratus* ♀ × *Dendraster* ♂ hybrids were strictly maternal in terms of rate of development both before gastrulation and up to the pluteus stage; *Den-*

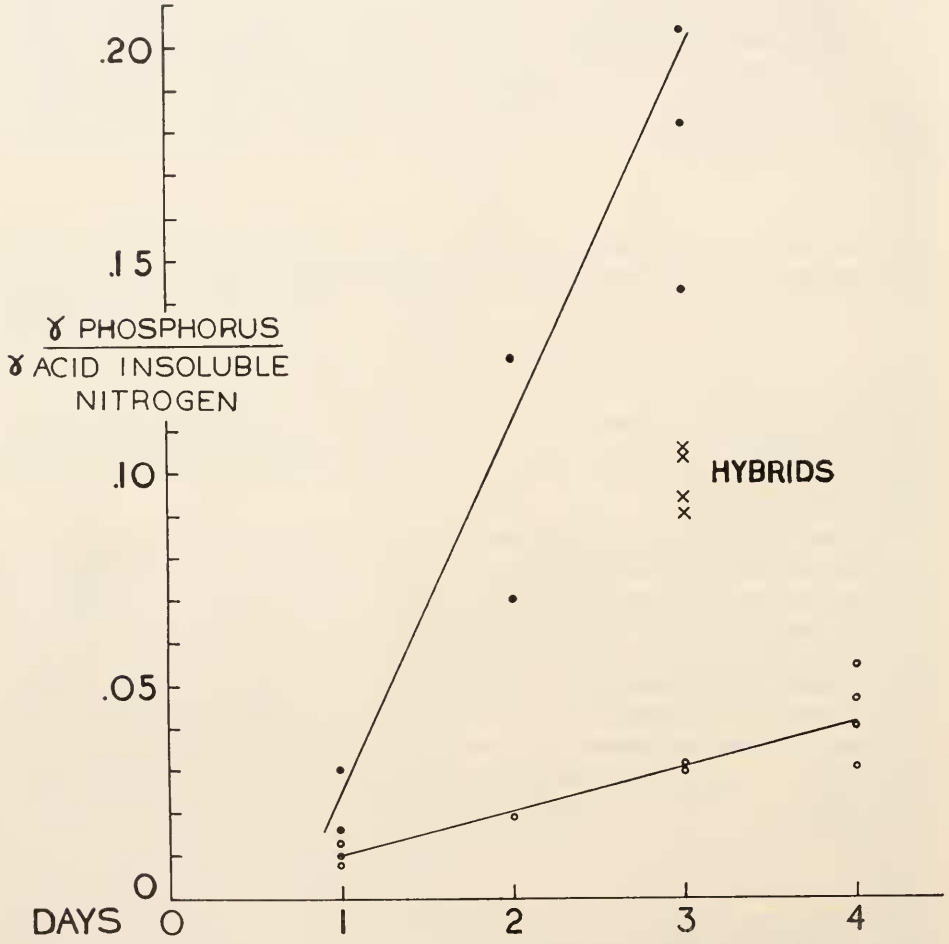

FIGURE 1. Alkaline phosphatase activity of *Strongylocentrotus purpuratus, Dendraster excentricus* and *Strongylocentrotus purpuratus* ♀ × *Dendraster excentricus* ♂ hybrids. Expressed as the ratio of micrograms phosphorus released in a two-hour period per microgram of acid-insoluble nitrogen. Solid dots indicate determinations upon *Dendraster*, open circles are for *S. purpuratus* and × indicates determinations upon the hybrids.

draster reaches the pluteus stage three days after fertilization whereas *S. purpuratus* takes four days. The day after fertilization (first day) the hybrids were early blastulae, the next day (second) they were hatched swimming blastulae or early gastrulae, the third day they had reached the prism stage and on the fourth day after fertilization they were plutei. In terms of morphological appearance the hybrid

plutei look very much like those of *S. purpuratus*. They lack the extended oral and anal arms of the *Dendraster* plutei and the skeletal rods are located as they are in *S. purpuratus* plutei.

Alkaline phosphatase determinations in the *S. purpuratus* ♀ × *Dendraster* ♂ hybrids were routinely made on the third day after fertilization when the hybrids had reached the prism stage. Assays were made at this time (third day) since no estimations were made upon *Dendraster* plutei after this period, and also by this third day there was a marked disparity between the alkaline phosphatase activity of the two homologous species which is not true at the earlier stages when alkaline phosphatase activity of the two species is more similar. The enzyme estimations revealed values intermediate to those of either homologous species (Fig. 1) at a similar time after fertilization. These hybrid prism activities were not only greater than those of *S. purpuratus* prisms but also distinctly higher than the values obtained for four-day *S. purpuratus* plutei. This indicates that alkaline phosphatase activity is not merely reaching an appropriate level for a given stage of development, as is the case with so many metabolic activities. These data are interpreted as an elevation of alkaline phosphatase activity due to the presence of *Dendraster* nuclear material in the developing embryos. It is not known if this nuclear stimulation of alkaline phosphatase indicates a nuclear localization of the enzyme, but it certainly seems to demonstrate a quantitative nuclear control for this enzyme with the intermediate value resulting from the action of both maternal and paternal nuclear elements.

Several attempts to induce higher acid and alkaline phosphatase activities in *S. purpuratus* embryos were carried out by adding sodium β glycerophosphate (final concentration 0.1 M) to several of the cultures at the mesenchyme blastula stage. The blastulae were allowed to develop for two days in the sea water containing the substrate and then acid and alkaline phosphatase assays were made at the pluteus stage. However, in two sets of experiments involving such cultures and their controls (where no substrate was added) no increase in activity was noted. Usually the activity of the controls was slightly higher than that of the cultures to which glycerophosphate had been added.

In order to determine if inadequate homogenization might account for the low alkaline phosphatase values, *S. purpuratus* embryos were frozen and thawed several times before homogenization so as to facilitate rupture of the cells. This procedure had no effect upon alkaline phosphatase activity since similar values were obtained for fresh material, once frozen, and twice frozen samples. Microscopic examinations of the homogenates were made routinely in all experiments. In all cases no large clumps of cells were observed, but undoubtedly not all cells were broken by the homogenization. This does not affect the interpretation of the results since the embryos of *S. purpuratus, Dendraster* and the hybrids were disintegrated in the same manner, and an equal degree of homogenization apparently was obtained in each case. It might also be expected that the hybrids would yield homogenates much like those of *S. purpuratus* since the only *Dendraster* component of the hybrids is nuclear and hence they are probably of equal fragility.

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Summary

1. Alkaline phosphatase estimations upon sea urchin embryos (*S. purpuratus*) showed a slow rate of increasing activity up to the pluteus stage while sand dollar embryos (*Dendraster excentricus*) showed a very rapid increase up to this same stage.

2. Hybridization between *Strongylocentrotus purpuratus* ♀ and *Dendraster excentricus* ♂ resulted in alkaline phosphatase activity at the prism stage which was intermediate between that of the two homologous species and higher than that of the maternal species. This is interpreted as indicating a nuclear control of alkaline phosphatase activity.

3. Addition of substrate (0.1 *M* sodium β glycerophosphate) to cultures of developing *S. purpuratus* embryos did not affect an increase in acid or alkaline phosphatase activity.

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